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## INTRODUCTION:

Despite intense research efforts, cancer remains the second leading cause of death in the United States. Mortality is seldom caused by primary tumors, but rather by the effect of metastases on distant organs. The lymphatic system serves as a common route of metastasis for many cancers of epithelial origin. There is growing evidence that lymphangiogenesis, the sprouting of new lymphatics from pre-existing lymphatics, facilitates the dissemination of cancer. Interestingly, the growth factor VEGF-A stimulates lymphangiogenesis; however, the underlying mechanisms have not been fully defined. To achieve a better understanding of VEGF-A's function in biology, our lab helped develop a novel anti-VEGF-A antibody (r84) that specifically blocks mouse and human VEGF-A activation of VEGFR2, but not VEGFR1. The aims of this proposal use r84 to 1) delineate the cellular and molecular mechanisms underlying VEGF-A-induced lymphangiogenesis and 2) characterize the effect of anti-VEGF-A therapy on the lymphogenous spread of breast cancer.

## BODY (TRAINING ACCOMPLISHMENTS):

Every postdoctoral scholar at UT Southwestern Medical Center must participate in the postdoctoral training program. I recently completed the Research Track of postdoctoral training which included courses on ethics, public speaking, and individual development. Additionally, completion of the training program required participation in journal clubs and mentored research. Moreover, I supplemented my training by participating in the multidisciplinary breast conference at UT Southwestern Medical Center. During this weekly conference, clinicians that treat patients with breast cancer present interesting observations from the clinic. These presentations cover histological and imaging findings as well as response to therapy. Attending this weekly conference has increased my understanding of the successes and hurdles in breast cancer research and in the clinic.

## BODY (RESEARCH ACCOMPLISHMENTS):

### ***VEGFR2 and VEGFR1 Expression by Lymphatic Endothelial Cells***

Several techniques were performed to demonstrate VEGFR2 expression by lymphatic endothelial cells (LECs). Purified primary human dermal lymphatic endothelial cells (HDLECs) were used to show VEGFR2 expression *in vitro* (Fig 1A-C, Appendix). Reverse-transcription PCR (RT-PCR) of cDNA generated from mRNA from PAE/FLT (negative control), PAE/KDR (positive control), and HDLECs revealed *VEGFR2* expression by HDLECs (Fig 1D, Appendix). Furthermore, VEGFR2 expression by HDLECs was demonstrated by Western blot analysis using lysates from the cell lines (Fig 1E, Appendix). VEGFR1 expression by HDLECs was also detected by RT-PCR and immunocytochemistry (data not shown).

Immunofluorescence staining of adult mouse ear skin revealed co-localization of the lymphatic marker podoplanin with VEGFR2 (Fig 1F-K, Appendix). Interestingly, VEGFR2 was enriched in lymphatic valves (Fig 1 I-L, Appendix). Lastly,  $\beta$ -galactosidase activity was observed for blood and lymphatic vessels in the skin of *Vegfr2<sup>+/-LacZ</sup>* following wholemount staining with X-gal (Fig 1M, Appendix). Taken together, these data are in agreement with other reports that lymphatic endothelial cells express VEGFR2 *in vitro* and *in vivo* (reviewed by Cueni and Detmar, 2008).

### ***Blocking VEGF-A Activation of VEGFR2 Suppresses Lymphangiogenesis (SOW Task#2)***

Adenoviral and transgenic overexpression of VEGF-A induces lymphangiogenesis in the skin of mice (Nagy et al., 2002; Hong et al., 2004). To determine whether VEGF-A activation of VEGFR2 or VEGFR1 regulates

lymphangiogenesis, we treated SCID mice bearing MDA-MB-231 tumors with r84, a fully human antibody that specifically blocks mouse and human VEGF-A from activating VEGFR2 but not VEGFR1. r84 effectively controlled tumor growth and angiogenesis (data not shown). Furthermore, r84 suppressed lymphangiogenesis in MDA-MB-231 xenografts (Fig 2A-C, Appendix). These data reveal that specifically blocking VEGF-A activation of VEGFR2 is sufficient to suppress lymphangiogenesis.

### ***VEGF-A Activation of VEGFR2, not VEGFR1, Drives HDLECs Proliferation and Migration (SOW Task#3)***

In order for lymphangiogenesis to occur, LECs must replicate, survive, and migrate to form a nascent vessel. To determine whether VEGF-A activation of VEGFR2 regulates these cellular processes, several *in vitro* experiments were performed with primary HDLECs. The addition of VEGF-A to reduced-serum media induced the proliferation of HDLECs as measured by the Cell Titer Blue assay (Fig 3A, Appendix). Interestingly, r84 blocked VEGF-A-induced proliferation whereas XTL (control IgG) had no effect (Fig 3A). VEGF-A also stimulated the migration of HDLECs, a process that was sensitive to r84 but not XTL (control IgG; Fig 3B, Appendix). These data indicate that VEGF-A activation of VEGFR2, not VEGFR1, drives LEC proliferation and migration.

### ***VEGF-A Promotes PKC Dependent Phosphorylation of ERK1/2 in HDLECs (SOW Task#3)***

To achieve a better understanding of how VEGF-A/VEGFR2 drives LEC proliferation and migration, the signal transduction pathways activated in primary HDLECs in response to VEGF-A were analyzed. The interaction of VEGF-A with VEGFR2 on blood endothelial cells (BECs) leads to receptor dimerization and phosphorylation on several tyrosine residues. These phosphorylated tyrosine residues regulate the kinase activity of VEGFR2 and serve as docking sites for adapter proteins that promote specific signal transduction cascades (Fig 4A, Appendix). VEGF-A stimulation of primary HDLECs results in the phosphorylation of Tyr 951, 1054, and 1175 (Fig 4B, Appendix). These results indicate that the phospho-tyrosine profile of activated VEGFR2 expressed by LECs is similar to that of BECs.

*In vitro* and *in vivo* studies have shown that Tyr 1175 is required for phospholipase-C gamma (PLC $\gamma$ ) recruitment and activation by VEGFR2 (Takahashi et al., 2001). Subsequently, PLC $\gamma$ /PKC signaling, rather than Ras, stimulates the activation of the MAPK ERK1/2 (Takahashi et al, 1999). Interestingly, signaling from Tyr 1175 but not Tyr 1214 is required for angiogenesis and survival in mice, suggesting that PLC $\gamma$ /PKC signaling serves a crucial role in VEGF-A induced angiogenesis (Sakurai et al., 2005). VEGF-A stimulation of primary HDLECs resulted in the phosphorylation of PLC $\gamma$  and ERK1/2 (Fig 4C, Appendix). Furthermore, VEGF-A-induced activation of ERK1/2 was dependent on PKC.

### ***VEGF-A-Induced HDLEC Proliferation and Migration is Mediated by ERK1/2 (SOW Task#3)***

ERK1/2 plays a critical role in promoting the proliferation and migration of different cell types in response to growth factors. To examine the role VEGF-A-induced activation of ERK1/2 serves in LECs, the MEK inhibitor PD098059 (PD) was used. PD effectively blocked VEGF-A activation of ERK1/2 and VEGF-A induced proliferation and migration of primary HDLECs (Fig 5A-C, Appendix). These results indicate that VEGF-A driven proliferation and migration of LECs is mediated by the MEK-ERK1/2 MAPK pathway.

Because VEGF-A activation of VEGFR2 regulates HDLEC proliferation and migration, and signaling through the ERK MAPK cascade controls these processes, the effect of r84 on ERK1/2 activation of was examined. Western blot analysis using phospho-specific antibodies revealed that r84 blocked VEGF-A activation of

VEGFR2, PLC $\gamma$ , and ERK1/2 (Fig 6, Appendix). These data reveal that VEGFR2, not VEGFR1, is the primary receptor regulating VEGF-A activation of PLC $\gamma$  and ERK1/2.

### ***Model for VEGF-A Regulation of Lymphangiogenesis***

Taken together, our results indicate that one mechanism by which VEGF-A induces lymphangiogenesis is by the activation of VEGFR2 on LECs. This results in ERK1/2 mediated LEC proliferation and migration, two cellular processes that serve critical roles in lymphangiogenesis (Fig 7, Appendix).

### ***Generation of Prox1-Cre transgenic mice***

VEGFR2 deficient mouse embryos die approximately 3-days before the lymphatic system develops. Therefore, the direct role VEGFR2 serves in formation of the lymphatic system and in tumor lymphangiogenesis remains unknown. To tackle this question we generated *Prox1-Cre* transgenic mice to cross with *Vegfr2<sup>flxed</sup>* mice. Offspring from this cross will exhibit a deficiency of VEGFR2 in lymphatics but retain expression in blood vessels.

Prox1 is a transcription factor expressed by lymphatic endothelial cells and not blood endothelial cells. We used a 4 kb region upstream of the start codon of Prox1 to drive Cre recombinase. This region was previously reported to drive GFP expression lymphatics in mouse embryos. Unfortunately, this 4 kb region was not strong enough to drive high levels of Cre recombinase in our transgenic mice. Recently, a paper was published reporting that COUP-TFII sites 10 kb upstream of the Prox1 start codon are required for Prox1 expression. Therefore, our transgenic mice may not work because they lack COUP-TFII sites. To overcome this problem, we are obtaining Prox1-Cre/GFP knock-in mice from Dr. Guillermo Oliver at St. Jude Children's Research Hospital.

### **KEY RESEARCH ACCOMPLISHMENTS:**

- Showed that specifically blocking VEGF-A activation of VEGFR2 with r84 is sufficient to suppress lymphangiogenesis in MDA-MB-231 xenografts.
- Demonstrated that VEGF-A activation of VEGFR2 but not VEGFR1 stimulates HDLEC proliferation and migration.
- Analyzed signal transduction pathways activated in HDLEC and showed VEGF-A induced phosphorylation of VEGFR2 on several key tyrosine residues, PLC $\gamma$ , and ERK1/2.
- Revealed that VEGF-A induced proliferation and migration are mediated by ERK1/2.
- Demonstrated that VEGF-A activation of VEGFR2 but not VEGFR1 drives phosphorylation of ERK1/2 in HDLECs.

### **REPORTABLE OUTCOMES:**

My DOD Postdoctoral fellowship has been acknowledged in a recently accepted review and a poster presentation.

### ***Review***

1. Witte MH, Dellinger MT, McDonald D, Boccardo F, Campisi C, Sleeman J, Gershenwald J.

2011. Lymphangiogenesis and Hemangiogenesis: Potential Targets for Therapy. *Journal of Surgical Oncology (In Press)*.

#### Poster Presentation

1. **Dellinger MT**, Dineen S, Roland C, Brekken RA. Inhibition of VEGF-A activation of VEGFR2 blocks lymphangiogenesis by preventing the activation of ERK. Metastasis and the Tumor Microenvironment. (September 12-15, 2010; Philadelphia, PA).

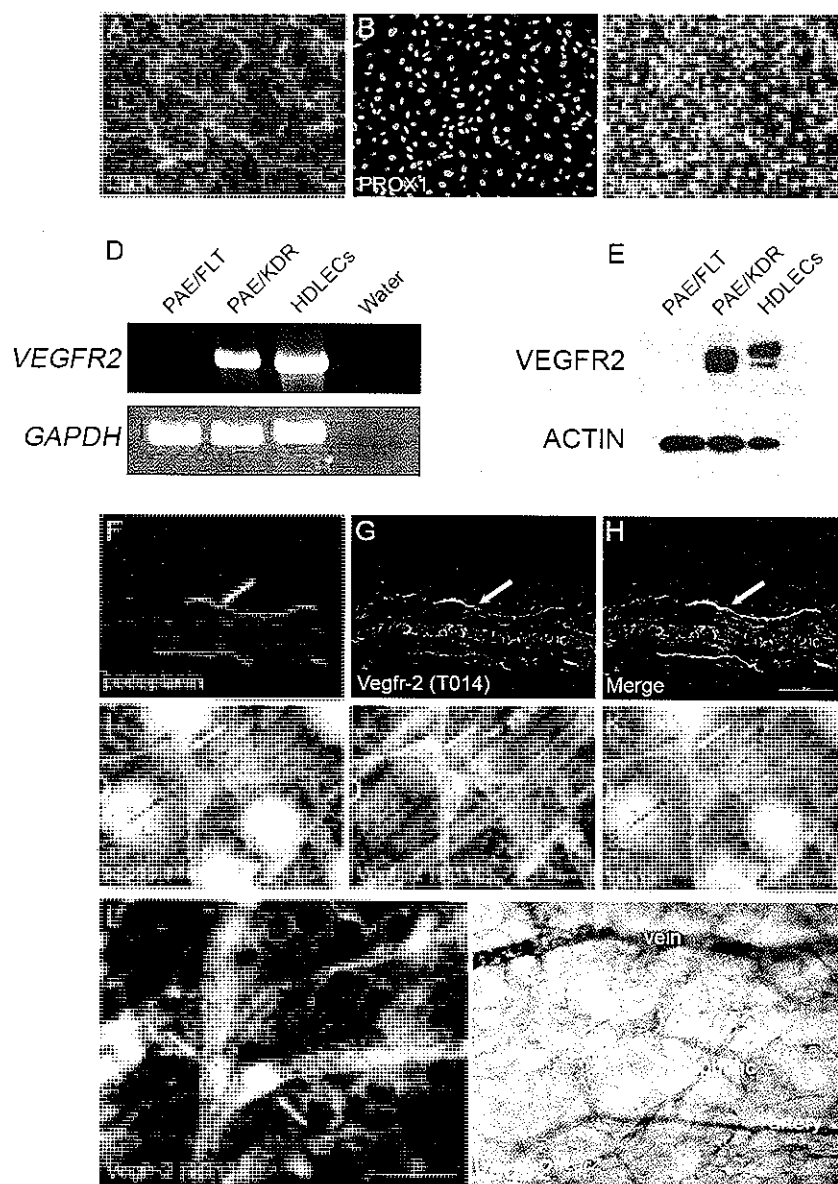
#### CONCLUSION:

In conclusion, we show for the first time that inhibition of VEGF-A activation of VEGFR2 on lymphatic endothelial cells suppresses lymphangiogenesis by blocking ERK1/2 driven LEC proliferation and migration. Furthermore, we demonstrate that although LECs are distinct from BECs, they respond to VEGF-A similarly. Therefore, therapeutic agents targeting the VEGF-A/VEGFR2 axis could be useful to prevent the pathological formation of blood and lymphatic vessels.

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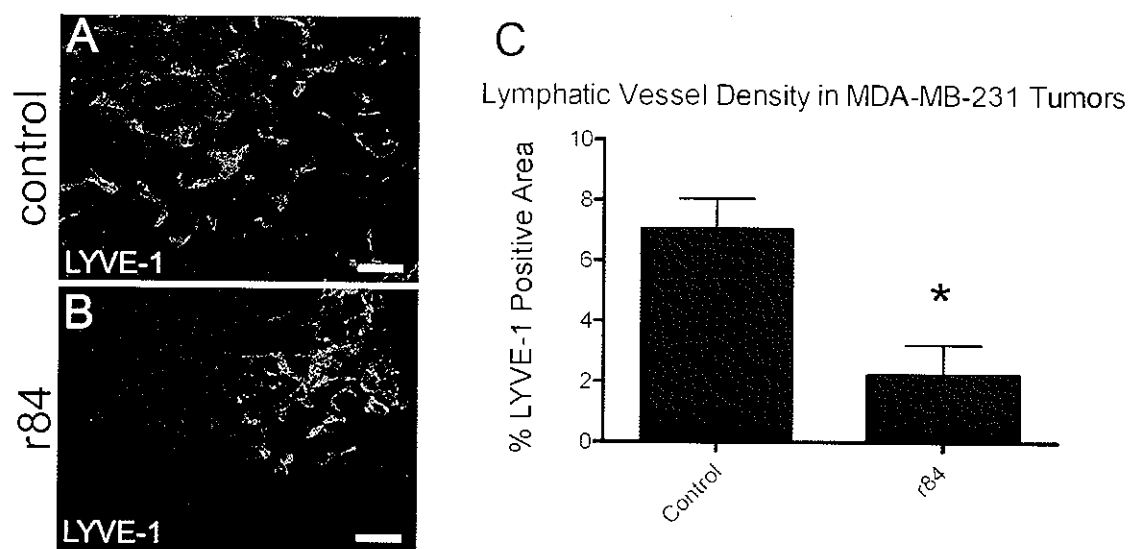
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## APPENDICECES

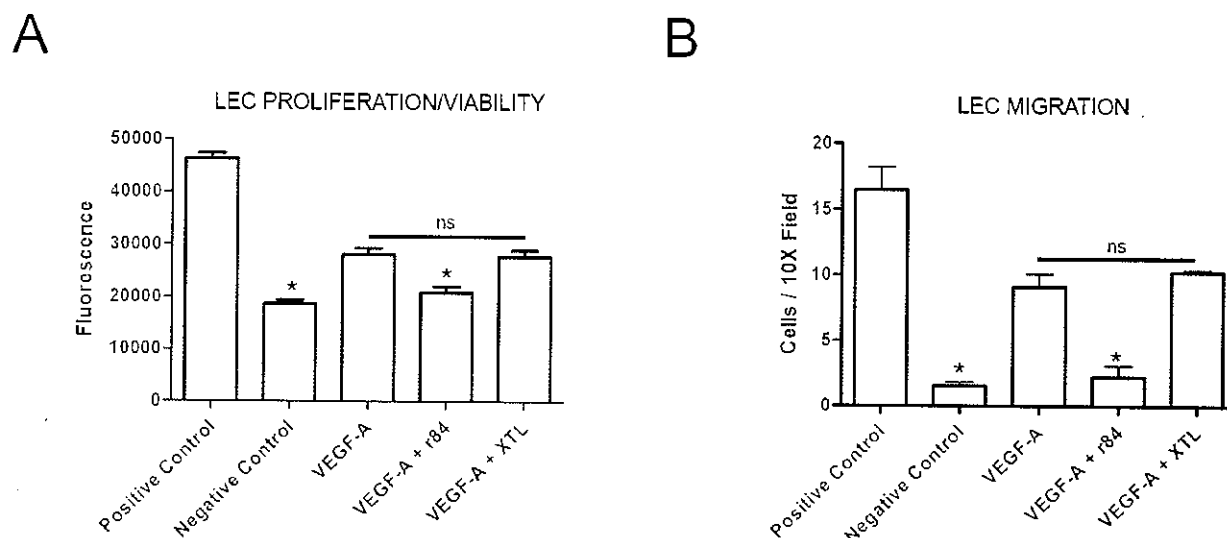


**Figure 1. VEGFR2 expression by lymphatic endothelial cells in vitro and in vivo. A-C:** Immunocytochemistry demonstrating that primary human dermal lymphatic endothelial cells (HDLECs) purchased from LONZA express the endothelial cell marker CD31 and lymphatic marker PROX1. **D:** Reverse-Transcription PCR results showing HDLECs expression of *VEGFR2*. PAE/FLT cells are porcine aortic endothelial cells that express VEGFR1 but not VEGFR2 (negative control). PAE/KDR cells are porcine aortic endothelial cells that express VEGFR2 but not VEGFR1 (positive control). **E:** Western blot results demonstrating VEGFR2 expression by HDLECs and PAE/KDRs (positive control) but not by PAE/FLT (negative control). **F-H:** Immunofluorescence staining of ear skin for the lymphatic marker podoplanin (green) and Vegfr-2 (red) shows that lymphatics (arrows) and blood vessels express Vegfr-2. **I-L:** Wholemount immunofluorescence staining of ear skin reveals that Vegfr-2 is enriched in collecting lymphatic vessels and valves (arrows). **M:** β-galactosidase activity is present in dermal lymphatics (arrow) in newborn *Vegfr2<sup>+/lacZ</sup>* mice.



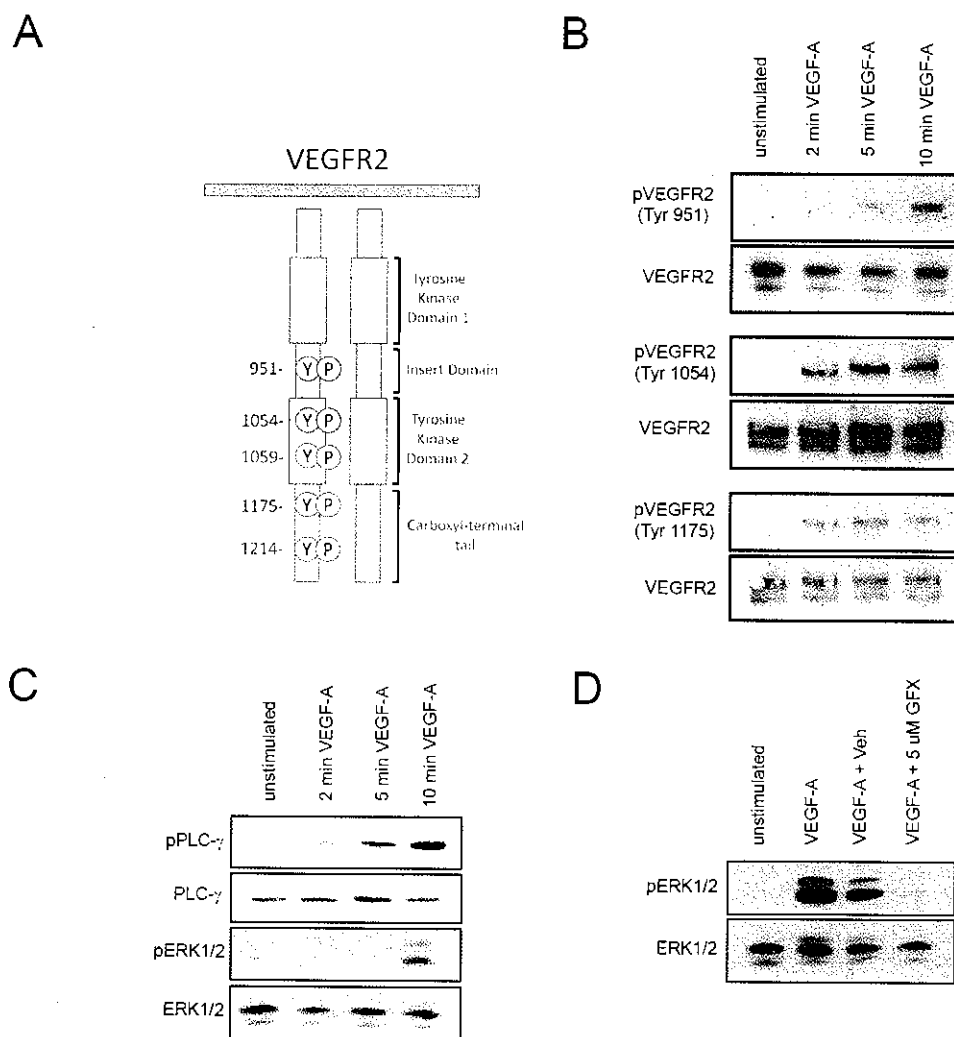


**Figure 2. Lymphatic vessel density in r84 treated tumors is significantly lower than control tumors.** **A,B:** Immunofluorescence staining of frozen MDA-MB-231 tumor sections for the lymphatic marker LYVE-1 identified lymphatic vessels in control IgG (A) and r84 (B) treated tumors. **C:** To determine whether the density lymphatic vessels in control and r84 treated tumors was different, the entire area of each LYVE-1 stained tumor section was examined at low magnification and the percent of LYVE-1 positive area was determined for each field using NIS-Elements imaging software. The 10 fields with the highest LYVE-1 positive percent area were averaged together to yield a final score for each tumor and group means were tested for significance by an unpaired student's t-test. The percent of LYVE-1 positive area of control tumors ( $7.03 \pm 1.013$ ) was significantly greater than r84 treated tumors ( $2.23 \pm 0.986$ ). Scale bars in A and B = 100  $\mu$ m. C mean  $\pm$  SE; Asterisk =  $P = 0.0042$ .

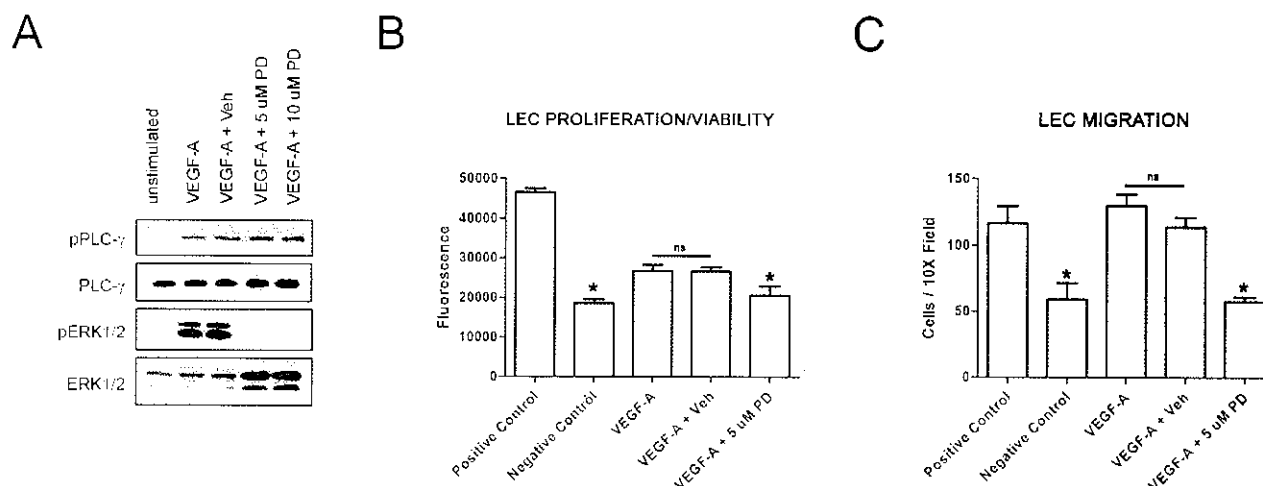


**Figure 3. r84 blocks VEGF-A induced lymphatic endothelial cell proliferation/viability and migration.**

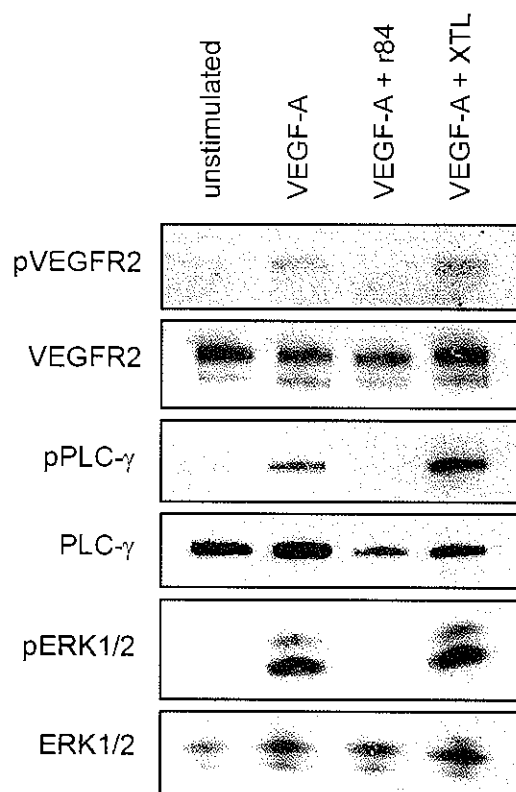
**A:** A proliferation/viability assay was performed with the Cell Titer Blue reagent (Promega). In this assay, viable cells convert Resazurin to the fluorescent compound Resorufin. Fluorescence is then measured with a plate reader as a surrogate for the number of cells. VEGF-A (100 ng/ml) enhanced the proliferation/viability of lymphatic endothelial cells. r84 (500 molar excess) blocked VEGF-A-induced viability of lymphatic endothelial cells whereas XTl (control IgG, 500 molar excess) had no effect. Fluorescence was measured following 48 hours of exposure to the various experimental conditions. **B:** Lymphatic endothelial cells migrated toward VEGF-A (100 ng/ml) in an overnight transwell migration assay. r84 (500 molar excess) blocked VEGF-A induced migration of lymphatic endothelial cells whereas XTl (control IgG, 500 molar excess) had no effect. Significance tested by ANOVA. Asterisk  $P < 0.05$ . ns = not significant.



**Figure 4. VEGF-A stimulation of LECs leads to the phosphorylation of VEGFR2, PLC $\gamma$ , and ERK1/2.** **A:** Diagram depicting phosphorylation sites of the intracellular domain of VEGFR2. **B,C:** Lysates of primary human dermal LECs were made after stimulating LECs with recombinant human VEGF-A (100 ng/ml) for 2, 5, or 10 minutes. The activation of VEGFR2, PLC $\gamma$ , and ERK1/2 was detected by Western blotting using phospho-specific antibodies. **D:** LECs were pretreated with DMSO (Veh) or the PKC inhibitor GF10203X for one hour prior to 10 minute stimulation with VEGF-A (100 ng/ml). ERK1/2 activation was detected by Western blotting using an anti-phospho-ERK1/2 antibody.



**Figure 5. PD098059 (PD) blocks VEGF-A induced activation of ERK1/2 and proliferation and migration of LECs.** **A:** Lysates were generated of LECs treated with: 1) VEGF-A (100 ng/ml, 10 minutes), 2) DMSO + VEGF-A (100 ng/ml, 10 minutes), or VEGF-A (100 ng/ml, 10 minutes) following 1 hour pre-treatment with the MEK inhibitor PD098059 (PD). ERK1/2 activation was detected by Western blotting using an anti-phospho-ERK1/2 antibody. PD blocked VEGF-A activation of ERK1/2 in LECs. **B:** To determine the effect of PD on VEGF-A induced proliferation/viability of LECs, a viability/proliferation assay was performed. To this end, LECs were cultured in the presence of VEGF-A (100 ng/ml) with or without 5 μM of PD. Cell titer blue reagent (Promega) and a fluorescent plate reader were used to determine the amount of viable cells after 48 hours. PD effectively blocked VEGF-A induced proliferation/viability of LECs. **C:** LECs were pre-treated with DMSO (Veh) or 5 μM of PD for one hour. During this time, the experimental conditions were added to the wells of a 24-well plate. Subsequently, primary HLDECs were seeded in the upper chamber of an insert and allowed to migrate overnight. PD blocked VEGF-A-induced migration of HDLECs. Significance tested by ANOVA. Asterisk  $P < 0.05$ . ns = not significant.



**Figure 6. r84 blocks VEGF-A induced activation of ERK1/2 in LECs.** Lysates were generated of LECs stimulated with VEGF-A (100 ng/ml, 10 minutes) in the presence or absence of r84 (500 molar excess) or XTL (control IgG, 500 molar excess). The activation of VEGFR2, PLC $\gamma$ , and ERK1/2 was detected by Western blotting with phospho-specific antibodies. Total VEGFR2, PLC $\gamma$ , and ERK1/2 was measured to control for loading.

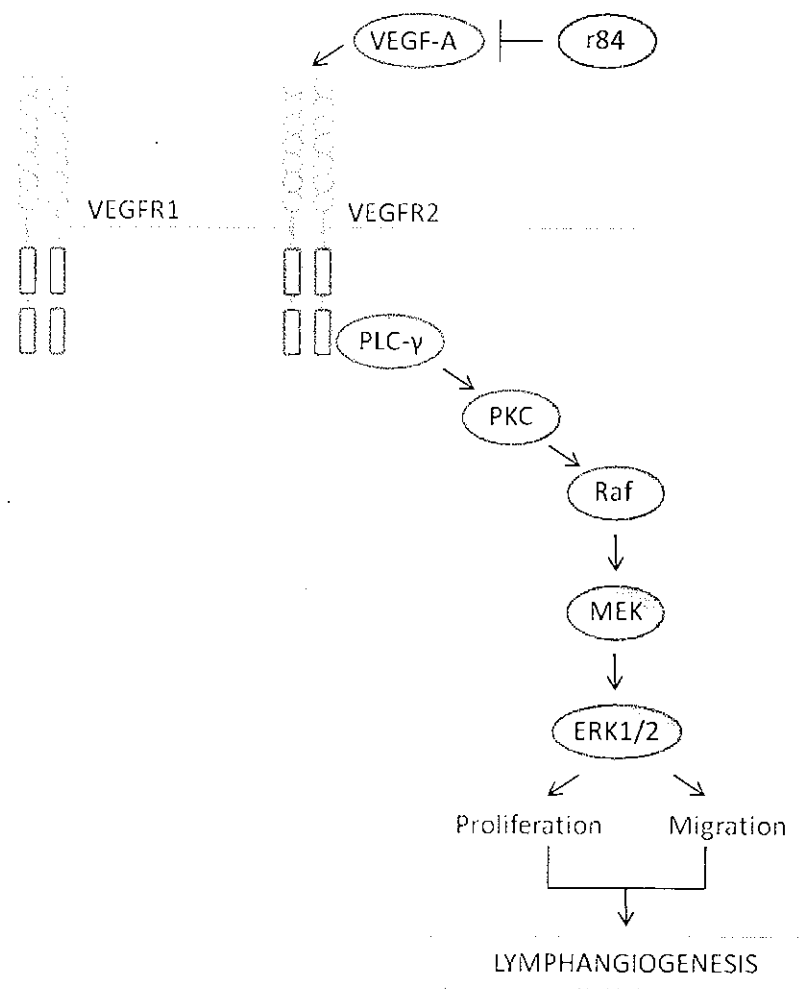


Figure 7. Model for r84 mediated inhibition of lymphangiogenesis. .